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(54) Title: METHOD OF PRODUCING A THYMIC MICROENVIRONMENT THAT SUPPORTS THE DEVELOPMENT OF DENDRITIC CELLS

(57) Abstract

The present invention relates, in general, to thymic microenvironment and, in particular, to a method of producing a thymic microenvironment. The invention further relates to a method of treating congenital and acquired immunodeficiencies using a thymic microenvironment produced *in vitro*. Included in acquired immunodeficiencies are syndromes seen in malignant and autoimmune diseases as well as traditional T cell immunodeficiency diseases such as occurs in AIDS.

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METHOD OF PRODUCING A THYMIC MICROENVIRONMENT THAT SUPPORTS THE DEVELOPMENT OF DENDRITIC CELLS

This application claims priority from U.S. Provisional Application No. 60/031,445, filed October 10, 1996, the entire contents of which is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates, in general, to thymic microenvironment and, in particular, to a method of producing a thymic microenvironment *in vitro* that supports the development of dendritic cells from hematopoietic pregenitor cells. The invention further relates to a method of treating congenital and acquired immunodeficiencies, including malignant and autoimmune diseases and traditional T cell immunodeficiency diseases such as occurs in AIDS.

BACKGROUND

Dendritic cells are antigen presenting cells (APC) distributed widely in lymphoid and non-lymphoid tissues (Steinman et al, Advances in Experimental Medicine & Biology 329:1-9 (1993); Steinman, Experimental Hematology 24:859-62 (1996); Young et al, Stem Cells 14:376-87 (1996); Steinman et al, Immunological Reviews 156:25-37 (1997)). Several different subsets of dendritic cells have been demonstrated in peripheral blood, skin, lymphoid organs and thymus (Caux et al,

Blood Cell Biochemistry 7:263-301 (1996); Caux et al, J. Exp. Med. 184:695-706 (1996); Caux et al, J. Immunol. 155:5427-5435 (1995); Chu et al, Br. J. Cancer. Suppl 23:S4-10 (1994)). Dendritic cells posses a distinct morphologic appearance, express high levels of MHC class I and II and have a potent ability to process antigens and activate T cells (Wettendorff et al, Adv. Exp. Med. Biol. 378:371-374 (1995); Wu et al, J. Exp. Med. 184:903-11 (1996); Shortman et al, Ann. Rev. Immunol. 14:29-47 (1996); Res et al, J. Exp. Med. 185:141-51 (1997); Mommaas et al, Eur. J. Immunol. 25:520-5 (1995); Mackensen et al, Blood 86:2699-2707 (1995); Caux et al, J. Exp. Med. 180:1841-7 (1994)). They also appear to play a critical role in mounting effective immune responses against microorganisms, neoplasms, and transplanted organs and may play a vital role in the induction of tolerance (Steinman et al, Advances in Experimental Medicine & Biology 329:1-9 (1993), Steinman, Ann. Rev. Immunol. 9:271-96 (1991)).

Dendritic cells developing within the thymus appear to be biologically distinct from extrathymic dendritic cells (Shorman et al, Ciba Found. Symp. 204:130-41 (1997); Shortman et al, Adv. Exp. Med. Biol. 378:21-9 (1995); Saunders et al, J. Exp. Med. 184:2185-96 (1996); Ardavin et al, Nature 362:761-3 (1993); Ardavin et al, Immunology Letters 38:19-25 (1993); Ardavin et al, Eur. J. Immunol. 22:859-62 (1992); Suss et al, J. Exp. Med. 183:1789-96 (1996); Winkel et al,

Immunology Letters 40:93-9 (1994)). Bone marrow, peripheral blood and umbilical cord blood (UCB) hematopoietic progenitors cultured with GM-CSF, TNF-α, and other cytokines *in vitro* generate mixed colonies containing both monocytes and dendritic cells which typically express primarily myeloid cell markers (Caux et al, Blood Cell Biochemistry 7:263-301 (1996); Caux et al, J. Exp. Med. 184:695-706 (1996); Caux et al, J. Immunol. 155:5427-5435 (1995); Caux et al, Nature 360:258-61 (1992); Rosenzwajg et al, Blood 87:535-44 (1996); Szabolcs et al, Blood 87:4520-30 (1996); Young et al, J. Exp. Med. 182:1111-1119 (1995)). In contrast, thymic dendritic cells express molecules normally considered as markers of lymphoid cells (Steinman et al, Immunological Reviews 156:25-37 (1997); Shortman et al, Adv. Exp. Med. Biol. 378:21-9 (1995); Saunders et al, J. Exp. Med. 184:2185-96 (1996); Ardavin et al, Nature 362:761-3 (1993); Ardavin et al, Immunology Letters 38:19-25 (1993); Ardavin et al, Eur. J. Immunol. 22:859-62 (1992); Winkel et al, Immunology Letters 40:93-9 (1994); Li et al, Exp. Hematol 23:21-5 (1995); Maraskovsky et al, J. Exp. Med. 184:1953-62 (1996); Sotzik et al, J. Immunol. 152:3370-7 (1994); Ardavin et al, Immunology Today 18:350-61 (1997); Lafontaine et al, Cellular Immunology 142:238-251 (1992)). In addition, thymic dendritic cell progenitors have been reported to generate both lymphoid cells and thymic dendritic cells (Saunders et

al, J. Exp. Med. 184:2185-96 (1996); Ardavin et al, Nature 362:761-3 (1993); Li et al, Exp. Hematol 23:21-5 (1995); Shortman et al, Ann. Rev. Immunol. 14:29-47 (1996)). Shortman and his colleagues have shown that murine "low-CD4" precursors isolated from the thymus can develop into dendritic cells and lymphoid cells, but not myeloid cells, following *in vivo* injection into the thymus or *in vitro* culture under certain conditions (Saunders et al, J. Exp. Med. 184:2185-96 (1996); Ardavin et al, Nature 362:761-3 (1993); Li et al, Exp. Hematol 23:21-5 (1995); Shortman et al, Ann. Rev. Immunol. 14:29-47 (1996)). These observations indicated that thymic dendritic cells may be more closely related to lymphoid cells than extrathymic dendritic cells. The growth and development of intrathymic dendritic cells may also be governed by different cytokines than those important in the development of extrathymic dendritic cells. Saunders et al demonstrated that murine "low CD4" thymic precursors developed into thymic type dendritic cells *in vitro* with a combination of TNF-α, IL-1ß, IL-3, IL-7, and SCF (Saunders et al, J. Exp. Med. 184:2185-96 (1996)). In contrast, the generation of dendritic cells from peripheral blood and bone marrow progenitors required GM-CSF (Caux et al, Blood Cell Biochemistry 7:263-301 (1996); Caux et al, Nature 360:258-61 (1992); Reid et al, J. Immunol. 149:2681-2688 (1992); Santiago-Schwarz et al, J. Leukoc. Biol. 52:274-81 (1992);

Strunk et al, Blood 87:1292-1302 (1996)). Thymic dendritic cells may also have different functional properties than extrathymic dendritic cells. In particular, thymic dendritic cells are believed to participate in the process of T cell negative selection and tolerance induction within the thymus (Steinman et al, Immunological Reviews 156:25-37 (1997); Shortman et al, Adv. Exp. Med. Biol. 378:21-9 (1995); Colic et al, Develop Immunol. 5:37-51 (1996); Tanaka et al, Eur. J. Immunol. 23:2614-21 (1993); Douek et al, Internat. Immunol. 8:1413-20 (1996)). Taken together, these observations indicate that thymic dendritic cells constitute a subset of dendritic cells with distinct developmental, immunophenotypic and functional properties.

Currently, most studies evaluating thymic dendritic cell biology have utilized murine models, in part because human thymic dendritic cells have been difficult to isolate and culture efficiently. For example, while Barcena et al demonstrated that human fetal thymic organ cultures (FTOC) could support the development of human fetal liver $CD34^{+}lin^{-}$ cells into monocytoid cells which displayed dendritic cell morphology (Barcena et al, J. Exp. Med. 180:123-32 (1994)), too few putative dendritic cells were recovered to be fully characterized. In another study, Res et al demonstrated that individual human $CD34^{+}CD38^{dim}$ thymocytes could differentiate into both T

and NK cells in FTOC and develop into dendritic cells when cultured *in vitro* with GM-CSF and TNFα (Res et al, Blood 87:5196-206 (1996)). However, the role that the thymus played in mediating the development of dendritic cells from intrathymic or extrathymic progenitors remained unclear because extrathymic culture of $CD34^{+}CD38^{dim}$ cells was required to generate dendritic cells. Consequently, certain aspects of human thymic dendritic cell biology remain uncharacterized.

Prior to the present invention, no experimental systems existed, other than FTOC, that generated thymic dendritic cells *in vitro* in a thymic microenvironment. The present inveniton provides systems that can be used to generate thymic dendritic cells from hematopoietic progenitors.

OBJECTS OF THE INVENTION

It is a general object of the invention to provide a thymic microenvironment *in vitro*.

It is a specific object of the invention to provide a thymic microenvironment suitable for use in human thymic transplantation and in the generation of thymic dendritic cells.

It is another object of the invention to provide a method of treating a congenital or acquired immunodeficiency disease or disorder.

The foregoing objects are met by the present invention which provides a method of producing, *in*

vitro, a thymic microenvironment and a method of generating dentritic cells from hematopoietic progenitor cells using same.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-F. Generation of $CD1a^+$ cells from $CD34^+CD38^-lin-$ and $CD34^+CD38^+lin-$ umbilical cord blood cells by co-culture with human thymic stromal cells. $CD34^+CD38^-lin-$ and $CD34^+CD38^+$ lin- cells were separated from lin- UCB cells using fluorescence activated cell sorting (FACS) using the gates shown in Fig. 1A. A post sort analysis of the sorted $CD34^+CD38^+lin-$ cells (gate R1) is shown in Fig. 1B, and of the sorted $CD34^+CD38^-lin-$ cells (gate R2) is shown in Fig. 1C. After 21 day co-culture with mixed thymic stromal cells (predominantly TE cells with 5-30% TF), $CD34^+CD38^-lin-$ UCB cells expanded approximately 40-fold and acquired cell surface CD1a (Fig. 1F). $CD34^+CD38^+lin-$ UCB cells also acquired cell surface CD1a (Fig. 1E), but to a lesser extent than $CD34^+CD38^-lin-$ UCB cells co-cultured with thymic stroma. Data presented are representative of 5 experiments.

Figures 2A-2T. Phenotype of $CD1a^+$ cells derived from $CD34^+CD38^-lin-$ UCB cells cultured on thymic stromal monolayers for 21 days. Cells were processed for 2- or 3-color staining with CD1a conjugated to FITC or PE and monoclonal antibodies labeled with complementary fluorescent molecules (FITC, PE, or Cy) as indicated. Histograms show fluorescent intensity of test (thick line) and isotype-matched control (thin line) antibodies gated on $CD1a^+$ cells. Results are representative of more than 3 experiments for each antibody tested.

Figures 3A and 3B. $CD1a^+CD14^-$ cells generated *in vitro* from $CD34^+38^-lin-$ or $CD34^+CD38^+lin-$ UCB cells on thymic stromal cell monolayers are good stimulators in allogeneic mixed lymphocyte reactions (MLR). Irradiated $CD1a^+CD14^-$ and $CD1a^-CD14^+$ cells grown on thymic stromal cell monolayers for 21 days and separated by FACS were used to stimulate 1.5×10^5 allogeneic monocyte-depleted peripheral blood mononuclear cells at different responder to stimulator ratios. Shown is a representative experiment of two performed. Error bars are mean ± standard deviation of triplicate wells in the experiment shown. Shown in Fig. 1A is the thymidine uptake (in counts per minute less background of $CD4^+$ responder T cells alone) induced by $CD1a^+CD14^-$ and $CD1a^-CD14^+$ cells derived from

$CD34^+CD38^-lin$-UCB progenitors. $CD1a^+CD14^-$ cells induce significantly more proliferation in MLR than $CD1a^-CD14^+$ cells ($p<0.001$ at all stimulator:responder ratios). Fig. 1B shows the thymidine uptake induced by $CD1a^+CD14^-$ and $CD1a^-CD14^+$ cells derived from more mature $CD34^+CD38^+lin-$ UCB progenitors. $CD1a^+CD14^-$ cells generated from $CD34^+CD38^+lin-$ UCB are also good stimulators in MLR, but are less potent that those generated from $CD34^+CD38^-lin-$ progenitors ($p=0.001$). $CD1a^-CD14^+$ cells generated from $CD34^+CD38^-lin-$ progenitors are not potent stimulators in MLR.

Figures 4A-4F. DCs grown on thymic stroma acquire markers of differentiated DCs in response to TNF-α. Shown are the phenotypes of $CD34^+CD38^-lin-$ UCB cells cultured in thymic stromal monolayers for 3 weeks with (Figs. 4D-4F) and without (Figs. 4A-4C) stimulation with 10 ng/ml TNF-α for 48 hours prior to harvest. When compared to cells not treated with TNF-α, a higher percentage of TNF-α treated $CD34^+CD38^-lin-$ cells express markers of DC including CD86, CD80 and CD83. Data are representative of 2 experiments performed.

Figures 5A-5D. Human thymic epithelial (TE) cells and thymic fibroblasts (TF) cultured in an artificial capillary system aggregate into nodules that recapitulate the human thymic microenvironment. Human

TE cells and TF from postnatal thymus were cultured *in vitro* and mixed at a ratio of 95 TE:5 TF in an artificial capillary system. Over a two to six week period, the TE and TF aggregated into nodules containing TE cells in an intertwined pattern encapsulated by fibroblasts (N=10). Shown are low power (Fig. 5A and Fig. 5B) and high power (Fig. 5C and Fig. 5D) photomicrographs of a representative nodule stained by indirect immunofluorescence with anti-keratin mAb AE-3 (Fig. 5A and Fig. 5C) and anti-stromal mAb TE7 (Fig. 5B and Fig. 5D). The arrowheads point to the fibrous capsule surrounding the nodule and the arrows point to a rest of thymic epithelium forming a rosette in sequential sections. Data are representative of 10 experiments.

Figures 6A and 6B. Umbilical cord blood progenitor cells differentiate into $CD1a^+$ cells with dendritic morphology in thymic nodules *in vitro*. Shown is the reactivity of anti-CD1a mAb Na1/34 in sections of thymic stromal nodules (Fig. 6A) cultured in media alone or (Fig. 6B) co-cultured with lin(-) umbilical cord blood (UCB) cells for 4 weeks. Note that the thymic stromal nodules co-cultured with UCB cells contain numerous $CD1a^+$ cells with a dendritic morphology while the thymic stromal nodules cultured in media alone do not contain any $CD1a^+$ cells. Dendritic processes of a single $CD1a^+$ cell in Fig. 6B are

indicated by arrows. The CD1a^{+} cells were cytoplasmic CD3-, CD33^{lo} and CD83-, which is consistent with the interpretation that these cells are early dendritic-lineage cells. Data are representative of 3 experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an *in vitro* method of producing a thymic microenvironment from cultured thymic cells and to a method of reconstituting the immune system of a mammal, for example, a mammal suffering from an immunodeficiency, using same. The invention also relates to a method of generating thymic dendritic cells from hematopoietic cells using such a thymic microenvironment.

Thymic epithelial cells suitable for use in the production of the thymic microenvironment of the present invention can be cultured from either fresh or frozen fetal or postnatal thymic tissues. The thymic epithelial cells can be cultured and isolated as described, for example, by Singer et al, Human Immunol. 13:161 (1985) and Singer et al, J. Invest. Dermatol. 92:166 (1989). Culture medium can contain any of a variety of growth factors, such as EGF, FGF, IGF, and TGF α/ß and insulin, and/or cytokines, such as IL-6, IL-8 and IFN-γ. Cells so derived can be used

immediately or stored frozen, for example, in a medium containing a cryoprotectant such as DMSO.

As indicated above, the present invention relates, in one embodiment, to a method of producing a thymic microenvironment from thymic stroma, particularly, human thymic stroma, and to a method of using same to support the development of primative hematopoietic stem cells into dendritic cells. In accordance with this embodiment, thymic stromal cells (thymic fibroblasts and thymic epithelial cells) obtained from human thymus tissue as decribed above are depleted of T cells, for example, by adding hydrocortisone to the culture medium (Singer et al, Human Immunol. 13:161 (1985)) or other T cell depleting agent such as deoxyguanosine (Hong, Clin. Immunol. Immunopathol. 40:136 (1986)), and extensive washing. Reduction in the number of thymic fibroblasts relative to thymic epithelial cells can be effected, for example, by complement-mediated lysis and/or growth on a feeder layer of irradiated NIH 3T3 fibroblasts.

Monolayers of thymic stromal cultures prepared as described above can be used directly for dendritic cell production or the thymic stromal cells can be cultured, for example, in an artificial capillary system (eg optionally, with a coating of ProNectin™ F), to provide 3-dimension cell aggregates or nodules (see Example 1). In addition to the technique described in Example 1, various zero gravity culture strategies or

strategies providing for three-dimensional cell aggregation with low to no shear stress can also be used to optimize thymic cell growth. Rotating-wall vessel (RWV) technology (Schwartz et al, J. Tiss. Cult. Meth. 14:51 (1992); Tsao et al, The Physiologist 35:549 (1992)) has shown three dimensional growth of many epithelial cell types including ovarian cancer cells (Becker et al, J. Cell. Biochem. 51:283 (1993)), primary salivary gland cells (Lewis et al, J. Cell Biochem. 51:265 (1993)), normal small intestine epithelium (Goodwin et al, Proc. Soc. Exp. Biol. Med. 202:181 (1993)), and embryonic kidney cells (Goodwin et al, J. Cell Biochem 51:301(1993)). Since RWV technology maintains low shear while simulating microgravity compared, for example, to impeller-stirred biorectors (Spaulding et al, J. Cell. Biochem. 51:249 (1993)), this system can be used for *in vitro* growth of functional thymic stroma suitable for engrafting. (Preliminary data suggest that proliferation and tissue formation in the spinner culture system (impeller-stirred biorector) may be limited by shear forces as increased shear forces (>5-7 dynes/cm^2) resulted in decreased aggregation of and cellular destruction on microcarriers).

Stem cells suitable for coculture with the thymic stromal cultures described above can be obtained from human umbilical cord blood, bone marrow and GCSF mobilized peripheral blood stem cells (Siena et al,

Exp. Hem. 23:1463 (1995)) (G-PBSCs). Preferred stem cells are $CD34^+CD38^-lin^-$ or $CD34^+CD38^+lin^-$. Such cells can be isolated from the indicated sources using commercially available lineage depleting antibody cocktails and art recognized cell sorting techniques (see Example 1).

The data presented in Example 1 demonstrate that umbilical cord blood $CD34^+CD38^-lin^-$ and $CD34^+CD34^+lin^-$ hematopoeitic progenitor cells cocultured on thymic stromal monolayers in serum free medium develop into cells with phenotypic, morphologic and functional characteristics of thymic dendritic cells. As also described in Example 1, thymic nodules produced as indicated above can support development of dendritic cells from hematopoietic cell progenitors by incubating the nodules with the progenitor cells under conditions such that the progenitor cells migrate into and differentiate in the nodules.

It will be appreciated that the thymic stromal cells used in the production of a thymic microenvironment as described herein can be genetically engineered so as to express various factors (eg secreted or surface bound factors) such as CD40 ligand and flt-3-ligand which can increase the yield and activity of the resulting thymic dendritic cells. Retroviral vectors can be used to effect the genetic manipulation, as can a variety of other engineering techniques. In addition, the thymic stromal cells can

be selected/designed to express specific MHC molecules. The thymic stromal cells can be used as packaging systems for transfer of genetic materials into developing hematopoietic cells) (Liu et al, Cell 86:367 (1996)). Such cells can be used to reconstitute an immune system that is superior to that of the recipient, for example, in its ability to defend against infection (specific MHC molecules to defend against infection with HIV) or its resistance to infection (CCR5 mutations in preventing infection with HIV).

In another embodiment, the present invention relates to immortalized human thymic epithelial cells and to a method of producing same. The establishment of immortalized human thymic epithelial stroma and individual clonal lines derived from such stroma is useful for several reasons. A readily available and consistent source of stroma which supports human thymic dendritic cell generation reduces intra-experimental variation and improves the logistics of generating thymic dendritic cells for study. An immortalized thymic epithelial stromal line can also be extensively characterized, validated for clinical use and can be expanded to scale up the generation of thymic dendritic cells. In addition, identification of cytokines important in the process of thymic deficient cell generation is aided by the development of such a line.

In accordance with this embodiment of the present invention, human thymic epithelial cells in thymus chunks can be immortalized via retroviral vector gene transfer of the papilloma virus E6E7 genes (Furukawa et al, Am. J. Pathol. 148:1763 (1996); LePoole et al, In Vitro Cell. & Devel. Biol. Animal 33:42 (1997)) in order to increase the number of passages these lines can be propagated. One such line, designated TE750, was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA, on October 10, 1997, under the terms of the Budapest Treaty, and was given Accession No. ____.

In order to demonstrate that cells such as TE750 cells could support the generation of thymic dendritic cells, they were co-cultured with $CD34^+CD38^-lin^-$ cells derived from umbilical cord blood. $CD1a^+CD14^-HLA\ DR^+$ cells were generated indicating that immortalized thymic epithelial can also support the development of thymic dendritic cells from primitive progenitors. In order to demonstrate that these immortalized cells could support the generation of thymic dendritic cells through soluble factors, $CD34^+CD38^-lin^-$ cells were placed into the insert of Transwell cultures, separated from the thymic epithelial by a permeable membrane which precluded cell-cell contact. While the overall cell number was reduced compared to cells grown in standard thymic epithelial cell co-cultures, $CD1a^+CD14^-HLA^-DR^+$ cells could be readily identified in the

Transwell cultures. Similar observations were made with co-cultures containing primary non-immortalized thymic epithelial stroma. These findings indicate that soluble factors produced by both primary and immortalized thymic epithelial stroma support the development of thymic dendritic cells from primitive progenitors. The thymic stroma cells of the invention can be used as sources of such factors both defined and undefined. These findings also indicate that cell surface bound factors are important in amplifying the generation of thymic dendritic cells. C-kit ligand and flt-3-ligand are logical candidates to account for this activity (Siena et al, Exp. Hem. 23:1463 (1995); Szabolcs et al, J. Immunol. 154:5851 (1995)).

In another embodiment, the present invention relates to a method of treating or preventing an autoimmune disease. This embodiment of the invention results from the fact that thymic dendritic cells play a critical role in the negative selection of autoreactive T cells. Accordingly, dendritic cells pulsed with antigens that incite autoimmune disease, for example, diabetes or multiple sclerosis (or other autoimmune disease), can be used in treatment or prevention protocols.

Taking as an example diabetes, insulin and other islet cell autoantigens (including GAD65, GAD67 and ICA69) are expressed in the thymus and the intrathymic expression of insulin mRNA is regulated by a known

disease susceptibility locus for Type I diabetes (Atkinson et al, New Engl. J. Med. 331:1428 (1994); Pugliese et al, Nat. Genetics 15:293 (1997)). Thymic expression of these antigens can mediate the negative selection and tolerance of islet cell reactive T cells. For tolerance to occur, these antigens must be expressed at very high levels in any thymic stromal cell type or they must be expressed by cells specializing in negative selection (preferably, dendritic cells). In accordance with the present embodiment, dendritic cells propagated and pulsed *ex vivo* with a diabetes inciting antigen (such as insulin) can be used to prevent or treat Type I diabetes. Specifically, dendritic cells expanded from progenitor cells of preferably a patient's own bone marrow and pulsed with diabetes inciting antigen (or encoding sequence) (Khoury et al, J. Exp. Med. 182:357 (1995)) can be used to treat or prevent this disease. Such cells can be administered intravenously or intrathymically. A similar approach can be used to treat other autoimmune diseases. In the case of multiple sclerosis, for example, dendritic cells produced as described above can be pulsed with myelin basic protein.

In a further embodiment, the present invention relates to a method of producing a cancer vaccine using dendritic cells prepared as described herein. A variety of murine studies have demonstrated that

dendritic cells generated or isolated from the spleen or bone marrow, when pulsed with tumor antigens *in vitro* and inoculated into tumor bearing animals, serve as extremely effective cancer vaccines (Porgador et al, J. Exp. Med. 182:255 (1995); Mayordomo et al, Nat. Med. 1:1297 (1995); Boczkowski et al, J. Exp. Med. 184:465 (1996); Alijagic et al, Eur. J. Immunol. 25:3100 (1995); Bernhard et al, Can. Res. 55:1099 (1995); Yang et al, Cell. Immunol. 179:84 (1997); Lotze, Ann. Surg. 226:1 (1997); Engleman, Biol. Bone Marrow Transp. 2:115 (1996); Murphy et al, Prostate 29:371 (1996); Tjoa et al, Prostate 27:63 (1995); Vieweg et al, Surg. ?Oncol. Clin. N. A. 4:203 (1995); Nair et al, Inter. J. Can. 70:706 (1997)). More recently, several groups, have initiated clinical trials designed to determine whether dendritic cell vaccines can be administered safely and effectively. In most of these trials, the dendritic cells have either been isolated or generated from peripheral blood mononuclear cells (Morse et al, Ann. Surg. 226:6 (1997); Engelman, Biol. Bone Marrow Transp. 2:115 (1996)). Recently, several methods have been described for improving the yield, safety, and efficiency of generating dendritic cells from the peripheral blood including using serum free media, adding macrophage conditioned media, and collecting peripheral blood mononuclear cells after treatment with chemotherapy and/or cytokines (Morse et al, Ann. Surg. 226:6 (1997); Bender et al, J. Immunol. Meth. 196:121

(1996); Romani et al, J. Immunol. Meth. 196:137 (1996); Maraskovsky et al, J. Exp. Med. 184:1953 (1996)). Immature dendritic cells are preferred as the cellular platform in a dendritic cell vaccine (Morse et al, Ann. Surg. 226:6 (1997); Romani et al, J. Immunol. Meth. 196:137 (1997)) as immature dendritic cells are better antigen processors than mature dendritic cells. In accordance with this embodiment, immature thymic dendritic cells produced in accordance with the present invention are pulsed with a tumor antigen (eg, MAGE, CEA, and her-2/neu (Boon et al, J. Exp. Med. 183:725 (1996)), or nucleic acid (RNA or DNA) encoding same using, for example, standard techniques. The pulsed cells can be used in vaccination therapies to treat existing tumors or prevent tumor development in individuals at increased risk (Boon et al, J. Exp. Med. 183:725 (1996); Hsu et al, Nature Med. 2:52 (1996)).

In yet a further embodiment of the present invention, the thymic microenvironments produced as described above can be used in thymic transplantation for treating congenital and acquired immunodeficiencies including, but not limited to, DiGeorge syndrome, Ataxia-Telangiectasia and Nezelof's disease (congenital immunodeficiencies) and acquired immunodeficiency syndromes, such as AIDS and cancer after ablative chemotherapy (Mackall et al, N. Engl. J. Med. 332:143 (1995)). The present thymic microenvironments can be transplanted into patients by a variety of methods

including implantation into the omentum or readily accessible muscles including, but not limited to, the forearm, thigh and calf muscles.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLE I

Dendritic Cell Development in Human Thymic Stroma

Experimental Details

Thymic stromal cultures: Thymic epithelial (TE) cells and thymic fibroblasts (TF) were cultured by an explant technique and propagated in enriched medium containing 67% DMEM (Gibco BRL, Grand Island, NY), 22% F12 (Gibco BRL), 5% Fetal Clone II serum (HyClone, Logan, Utah), 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 11 ng/ml recombinant human epidermal growth factor (Collaborative Biomedical, Bedford MA), 0.18 µM adenine, 10^{-10} M cholera toxin (ICN Biomedicals, Aurora, OH), 0.25 µg/ml fungizone and 50 µg/ml genatmicin (TE medium) on irradiated NIH 3T3 fibroblast feeder layers as described (Singer et al, J. Invest. Dermatol. 92:166-176 (1989), Singer et al, Hum. Immunol. 13:161-76 (1985)). Human thymus tissue was

obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery. Thymic stromal cells (thymic fibroblasts and TE cells) were depleted of T cells by culture in TE medium, and extensive washing. Cells were either used immediately or stored frozen in 7.5% DMSO-containing medium prior to expansion and use for reconstruction of the thymic stromal microenvironment. Contaminating thymic fibroblasts were removed from TE cell monolayers by treatment with 0.02% EDTA in PBS followed by complement-mediated lysis with mAb 1B10, which binds to a cell-surface antigen on human fibroblasts (Singer et al, J. Invest. Dermatol. 92:166-176 (1989)). TE cell preparations were >95% positive for the keratin marker AE-3 and negative for CD1a, CD7 and CD14. For coculture with sorted cord blood CD34+CD38- cells, 2.5 x 10^5 TE cells were plated in 24 well plates on irradiated NIH 3T3 fibroblast feeder layers, and irradiated with 2500 cGy once cells became confluent. Thymic fibroblasts (TF) were obtained by an explant technique and grown in TE medium without an NIH 3T3 feeder layer. Typically, TF outgrew TE cells and were ~98% pure by the first passage. The TF cultures used in this study were $\geq$98% positive for M38 (procollagen), <2% positive for AE3 and negative for CD1a, CD7 and CD14.

Lineage depletion and stem cell isolation by fluorescence-activated cell sorting (FACS): Human umbilical cord blood (UCB) was obtained as discarded material from the Department of Obstetrics and Gynecology of Duke University Medical Center. The UCB used in these studies was collected in sterile bottles containing an anticoagulant citrate buffer and processed within 18 hours of collection. The blood was diluted 1:2 with Dulbecco's phosphate buffered saline (PBS) and red blood cells were agglutinated at room temperature using 1% Hespan (DuPont Pharma, Wilmington, DE). Non-agglutinated white blood cells were harvested and residual red cells were hemolysed at 37°C in 0.17 M NH_4Cl containing 10 mM Tris-HCl, pH 7.2 and 200 mM EDTA. For lineage depletions, the white cell fractions were brought to 6-8 $X10^7$ cells/ml in PBS containing 4% fetal calf serum (FCS) and were depleted through the addition of a commercial antibody cocktail and magnetic colloid as per the manufacturer's instructions ($CD34^+$ StemSep enrichment cocktail, StemCell Technologies, Vancouver, BC). The mixtures of cells, antibodies and magnetic colloid were cleared of lineage-marked cells over a column held in a wrap-around magnet. The cells that passed through the column (lin^- cells) were collected, washed in Dulbecco's modified MEM (DMEM) with 10% FCS and stored on ice.

For fluorescence activated cell sorting (FACS), lin^- cells were pelleted and resuspended in 100 µl

PBS/2% FCS, incubated with anti-CD34 and anti-CD38 for 20-30 minutes. After 3 washes in PBS/2% FCS, cells were sorted on a FACStar Plus cell sorter (Becton Dickinson) and collected in sterile polystyrene tubes containing 100% FCS. Post sort analysis was performed on the last 10% of cells which were collected in separate tubes and reanalyzed on the flow cytometer.

Coculture of sorted stem cell and thymic stromal monolayers: Sorted $CD34^{+}38^{-}$lin-or $CD34^{+}CD38^{+}$ lin- cells were added onto irradiated confluent thymic stromal monolayers at 10^3 to 10^4 cells/well and cultured in 1 mL of serum free media. This media was made with 80% IMDM (Gibco BRL) 20% BIT 9500 (StemCell Technologies), 1 mg/ml glutamine, 40 mg/L lipoprotein (Sigma) and 0.1% mercaptoethanol. Cells were fed thrice weekly by carefully removing 0.5 mL of supernatant and replacing it with fresh media.

Antibody reagents: mAbs to the following antigens were used for indirect immunofluorescence staining: P3x63/Ag8 (IgG1, from American Type Culture Collection (ATCC), Rockville, MD); CD1a (Na1/34, from Andrew McMichael) (McMichael et al, Eur. J. Immunol. 9:205-10 (1979)); CD2 (35.1, ATCC), CD3 (Leu4, Becton Dickinson, Mountain View, CA), CD4 (Leu3a, ATCC), CD7 (3A1e) (Haynes et al, Proc. Natl. Acad. Sci. 76:5829-33 (1979)), CD14 (LeuM3) (Dimitriu-Bona et al, J. Immunol. 130:145-52 (1983)), AE3 (keratin from TT Sun) (Woodcock-Mitchell et al, J. Cell. Biol. 95:580-88.

(1982)), 1B10 (fibroblasts) (Singer et al, J. Invest. Dermatol. 92:166-176 (1989)), M38 (C-terminal region of type I procollagen) (McDonal et al, J. Clin. Invest. 78:1237-44 (1986)), and fluorescein-conjugated goat anti-mouse Ig (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Directly-conjugated antibodies to the following antigens were also used for multicolor analyses of cell surface antigens: CD2 (leu5, FITC), CD3 (leu4, PerCP), CD5 (leu1, PE), CD7 (leu9, FITC), CD8 (SK1, FITC), CD11c (S-HCL-3, PE), CD14 (MfP9, FITC), CD16 (B73.1, FITC), CD19 (leu12, FITC), CD25 (2A3, FITC), CD33 (leuM9, PE), CD34 (HPCA2, FITC, PE and Cy), CD38 (leu17, PE), CD56 (leu19, PE), CD80 (L307.4, PE) HLA-DR (L243, FITC), IgG1 (X40, FITC and PE) from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA); CD1a (T6, PE), CD4 (T4, PE), and CD83 (HB15a, PE) from Coulter (Hialeah, FL); CD3 (UCHT1, Cy) from Immunotech, Inc. (Westbrook, ME); CD1a (HI149, FITC), CD2 (RPA-2.10, Cy), CD40 (5C3, FITC), CD86 (2331(FUN-1), FITC), CD95 (DX2, FITC), HLA A,B,C (G46-2.6, FITC) and IgG1 (MOPC-21, Cy) from PharMingen, Inc. (San Diego, CA); and CD8 (DK25, RPE-Cy5) and CD13 (F0831, FITC) from Dako Corporation (Carpenteria, CA).

Phenotypic analysis using flow cytometry: For FACS analysis of cultured cells, cells were gently resuspended to leave thymic monolayers undisturbed, pelleted and resuspended in 100 µl of PBS/ 4% FCS and

held on ice. Fluorescence-conjugated antibodies were added directly to the cell suspensions. Following incubations for 20-30 minutes at 4°C, the cells were washed 3 times in PBS/4% FCS. Where necessary, the cells were fixed in 1% formaldehyde in PBS/2% FCS. Irrelevant isotype-matched mAbs were used as negative control. Quantitation of the surface staining was performed on a FACScan and a FACScalibur (Becton-Dickinson) using a 488 argon laser for fluorescence excitation. Data was analyzed using CellQuest software (Becton Dickinson). In all experiments, cells stained with isotype-matched control antibodies were used to set cursors so that <1% of the cells were considered positive.

Microscopy: Sorted cells were centrifuged onto glass slides using a Shandon cytocentrifuge (Shandon Southern Instrument Co., Sewickley, PA) at 1000 RPM for 3 minutes. Cytospins were air-dried and stained with Wright Giemsa stain and examined by light microscopy. For transmission electron microscopy, thymic nodules and sorted cells were fixed with 2% glutaraldehyde in 150 nM sodium cacodylate buffer plus 2.5 mM $CaCl_2$, pH 7.2, washed, and embedded in 1% agar. After post fixation for one hour on ice with 2% osmium tetroxide plus 1% potassium ferrocyanide, blocks were washed with cacodylate buffer followed by 200 mM sodium acetate, pH 5.2. Samples were stained *en bloc* for one hour with 1% uranyl acetate in sodium acetate buffer. After

dehydration with ethanol, the pellet was infiltrated with and embedded in EMBED 812 (EM Sciences, Fort Washington, PA). 90 nm sections were cut on a Reichart Ultracut E microtome and stained with uranyl acetate, followed by Sato lead, washed and examined with a Philips EM300 electron microscope (Philips, Eindhoven, The Netherlands).

Mixed lymphocyte reactions: Allogeneic responder PBMCs (1.5×10^5) obtained from healthy donors were cultured in RPMI 1640 supplemented with 10% FCS or 10% human AB serum in 96 well U-bottom tissue culture plates. Irradiated (3500 rads) sorted CD1a+CD14- and CD1a-CD14+ cells were added in graded doses of $1.5x10^2$ (1:1,000) to $1.5x10^4$ (1:10) cells in a total volume of 200 µl. Cell proliferation after 96 hours was quantified by adding 1 µCi (37kBq) of [*methyl*^{3}H] thymidine (NEN-DuPont, Boston, MA) to each well. After 16 hours, the cells were harvested onto filters and radioactivity was measured in a scintillation counter with results presented as the mean cpm for triplicate cultures.

Development of human thymic stromal microenvironment nodules: Cultured thymic stromal cells were co-cultured in an artificial capillary sytem (Cellmax; Cellco, Inc., Germantown, MD) with a coating of ProNectin™ F to promote adhesion of stromal cells to the capillaries. 20-100 x 10^6 thymic stromal cells

(95% TE cells by reactivity with anti-keratin antibody AE-3 and 5% TF by reactivity with anti-procollagen antibody M38) were seeded per capillary module with an extracapillary space of 12 ml. TE medium (Singer et al, Hum. Immunol. 13:161-76 (1985)) was pumped through the capillaries at a rate of 10 ml/min. Within 2-6 weeks, 1-2 mm nodules were readily apparent by visual inspection in the extracapillary space of the capillary modules. Nodules were harvested by cutting the module with a sterile pipe cutter. The nodules were separated from the capillaries by scraping with a sterile rubber policeman, and washed with DME containing 5% FCS. Sorted $CD34^{+}38^{-}$lin- or CD34+CD38+ lin- cells at 10^{3} to 10^{4} cells/well were added onto 24 well plates containing approximately 10 micronodules/well and cultured in 1 mL of serum free media.

Results

CD34+ cord blood cells differentiate into CD1a+ cells on thymic stromal cell monolayers: In order to determine whether human thymic stroma could support the development of DCs from hematopoietic progenitors, $CD34^{+}CD38^{-}$ lin- (R2 in Fig. 1A) and $CD34^{+}CD38^{+}$lin- umbilical cord blood cells (R1 in Fig. 1A) were isolated by sterile cell sorting and co-culture with pre-established irradiated human thymic stromal monolayers (Herbein et al, Stem Cells (Dayt) 12:187-97 (1994), Terstappen et al, Blood 77:1218-27 (1991)).

Prior to co-culture, the sorted populations had greater than 98% purity (Figure 1B and 1C) and were >98% CD1a- (Figure 1D). Following co-culture with thymic stromal monolayers in serum free media for 21 days, $CD34^+CD38^-$ cells expanded 43 ± 17 fold (N=3) and the $CD34^+CD38^+$ cells expanded 32 ± 16 fold (N=3). UCB progenitors cultured in serum-free media alone did not expand nor change in morphology. Immunophenotypic analysis of co-cultured cells revealed the presence of a number of $CD1a^+CD14^-HLA\text{-}DR^+$ cells (Figures 1 and 2) similar to previous descriptions of human DCs (Caux et al, Blood Cell Biochemistry 7:263-301 (1996)). The percentage of $CD1a^+CD14^-$ cells generated from CD34+CD38- cells ranged from 5-15% (mean 8.2% N=3) and from CD34+CD38+ cells ranged from 2-10% (mean 4.8% N=3). The observation that CD1a+CD14- cells could be generated from both the CD34+CD38-lin- and CD34+CD38+lin-populations suggested that both of these cell types could develop into DCs in the thymic stroma monolayers.

Morphology of CD34+ cells expanded in thymic stroma: In order to confirm that the CD1a+ cells grown in thymic monolayers were DCs, $CD1a^+CD14^-$ cells generated after 21 days of culture from both CD34+CD38-lin- and $CD34^+CD38^+$lin- umbilical cord cells were isolated by FACS and examined by light and electron microscopy. $CD1a^-CD14^+$ cells were also sorted from

both cultures to serve as controls. Analysis of the sorted cells revealed a purity greater than 97%. By light microscopy, $CD1a^{+}CD14^{-}$ cells possessed a DC morphology with an irregular shape and multiple dendritic processes. Examination of the ultrastructure by EM showed that $CD1a^{+}CD14^{-}$ cells had euchromatic, lobulated or indented nuclei and a clear cytoplasm with rough endoplasmic reticulum and well-developed Golgi apparati. These cells did not contain Birbeck granules. These findings are consistent with these cells being mature thymic type DCs. In contrast, the control $CD1a^{-}CD14^{+}$ cells from both precursor types had the morphologic appearance of macrophages, with indented nuclei and foamy cytoplasm and no evidence of cytoplasmatic dendritic projections.

Immunophenotype of CD1a+ cells expanded in thymic stroma: In order to better characterize the DCs generated from UCB progenitors on thymic monolayers, extensive phenotypic evaluations were performed using multiparameter FACS analysis (Fig. 2). $CD1a^{+}$ cells generated on thymic stroma from CD34+CD38-lin- UCB cells were negative for surface CD3, CD8, CD19, CD25, CD34, and CD95, expressed CD2, CD4, CD11c, CD13, CD16, CD33, CD38, CD40, CD45, CD49e, CD80, CD83, CD86, MHC class I and MHC class II.

Ability of CD1a+CD14- and CD1a-CD14+ cells generated in thymic stroma to act as antigen presenting cells: In order to determine whether the putative DCs generated on thymic stroma were able to activate T cells, $CD1a^{+}CD14^{-}$ and $CD1a^{-}CD14^{+}$ cells were sorted by FACS and tested in allogeneic MLRs. $CD1a^{+}CD14$- cells were much more potent stimulators in the MLRs than $CD1a^{-}CD14^{+}$ cells (Fig. 3). Further, $CD1a^{+}CD14^{-}$ cells generated from CD34+CD38-lin- UCB cells were more potent stimulators of the MLR on a per cell basis than the $CD1a^{+}CD14^{-}$ cells generated from CD34+CD38+lin- cells (Fig. 3). This suggests that more primitive progenitors may not only generate larger numbers of DCs but that these DCs may be qualitatively different from DCs generated from more mature progenitors.

Effect of TNF-a on thymic DC: Analysis of CD1a and CD14 expression on CD34+CD38-lin- UCB progenitors co-cultured with thymic stroma revealed the presence of several phenotypically distinct populations of cells. One possible explanation for this observation is that the co-cultures contained DCs at multiple stages of development. To test this hypothesis, the co-cultures were treated for 48 hours with TNF-α (10 ng/ml), a previously described DC maturation factor (Caux et al, Blood Cell Biochemistry 7:263-301 (1996), Caux et al, J. Exp. Med. 184:695-706 (1996), Caux et al, Nature

360:258-61 (1992)). TNF-α treatment induced expression of CD1a, CD83, CD80 and CD86 on large numbers of cells derived from CD34+CD38-lin- progenitors (Fig. 45). In addition, most of these cells displayed a dendritic cell morphology. While TNF-α treatment of co-cultures established with $CD34^{+}CD38^{+}$lin- cells caused an increase in the fraction of cells with mature DC markers, not all cells expressed DC markers and a significant number of $CD1a^{-}CD33^{+}$ cells were also observed. This suggested that these cultures may have contained a significant fraction of non-DC myeloid cells. This was confirmed by light microscopic examination that revealed a number of myeloid lineage cells including neutrophils and macrophages at different stages of maturation in the CD34+CD38+lin- co-cultures treated with TNF-α.

Formation of thymic microenvironment nodules from cultured thymic epithelial cells and thymic fibroblasts: Since thymic stromal monolayers do not have the full differentiation capacity of reaggregation cultures such as that seen with fetal thymic organ cultures (Barcena et al, J. Exp. Med. 180:123-32 (1994), Res et al, Blood 87:5196-206 (1996), Spits et al, Blood 85:2654-70 (1995)), and due to the difficulties of obtaining sufficient human fetal thymus for studies, a culture system was developed to form

three-dimensional aggregates of cultured post natal TE cells and thymic fibroblasts. After 2-6 weeks of co-culture in an artificial capillary system, human thymic fibroblasts and TE cells aggregated to form 1-2 mm nodules with a morphology and phenotype consistent with a thymic stromal microenvironment devoid of hematopoietic cells (N=10) (Fig. 5). The nodules contained TE cells (keratin positive) in a fibroblast matrix (identified by TE7) that was encapsulated by a layer of procollagen-positive fibroblasts. By transmission electron microscopy, the thymic stromal nodules contained numerous desmosomes and hemi-desmosomes indicating that the epithelial cells within the nodules are able to interconnect and form a network similar to that seen in normal thymus (Haynes et al, J. Exp. Med. 159:1149-68 (1984), Haynes et al, J. Immunol. 132:2678 (1984)).

TE cells in nodules did not terminally differentiate as determined by lack of reactivity with mAbs STE1, STE2 and 11.24 (CD44v9) (Patel et al, J. Clin. Immunol;. 15:80-92 (1995)), nor did they form Hassall's bodies. This pattern is similar to that seen in the thymic stroma of patients with severe combined immunodeficiency (reviewed in Haynes et al, J. Exp. Med. 159:1149-68 (1984), Patel et al, Int. Immunol. 6:247-254 (1996)).

CD34+ cord blood cells differentiate in thymic nodules into CD1a+ cells with DC morphology: To test the functional status of the thymic nodules, an evaluation was made as to whether umbilical cord blood hematopoietic cell progenitors migrate into and differentiate in the nodules *in vitro*. Lin- UCB cells were incubated with thymic nodules in a 24-well flat-bottom plate in serum free medium at 37°C. After 28 days of co-culture with thymic nodules, the nodules were analyzed for markers of T and NK cells (CD1a, CD3, CD7), progenitor cells (CD33, CD34), myeloid cells (CD14) and DC (CD1a, CD83). Nodules cultured in the absence of UCB progenitor cells were also analyzed. No CD3 or CD7 expressing cells were detected in the nodules by indirect immunofluorescence. However, there were numerous $CD1a^{bright}$ cells with dendritic morphology in the nodules seeded with lin- UCB cells (Fig. 6B), but not in the nodules cultured without UCB cells (Fig. 6A). The $CD1a^{bright}$ cells were $CD33^{lo}$ and $CD83^{-}$. Further, at day 0, lin^{-} cells did not express CD1a, suggesting that $CD1a^{+}$ cells resulted from the differentiation of progenitor cells within the nodule and not from spontaneous expansion of dendritic cells contaminating the lin^{-} population. Taken together, these findings indicate that progenitor cells migrated into the thymic nodules, and that thymic stromal

nodules were able to support CD34+lin- UCB cell development into DCs.

EXAMPLE 2

The slow turning lateral vessel (STLV) (Synthecon, Inc., Friendswood, TX) can be used to assess the utility of low fluid shear forces (typically 0.81 dyn/cm^2; Tsao et al, The Physiologist 35:549 (1992)) on the growth and differentiation of TE cells and thymic fibroblasts (TF). The STLV, filled with GM (Table 1) can be inoculated with Cytodex-3 microcarrier beads (collagen-coated dextran, Pharmacia) at a concentration of 10 cells/bead. After attachment of either TF or TE cells to the beads, the vessel can be rotated at calculated shear forces ranging from 0.51 dyn/cm^2 to 0.92 dyn/cm^2 (Tsao et al, The Physiologist 35:549 (1992); Goodwin et al, Proc. Soc. Exp. Biol. Med. 202:181 (1993)) for 7d. Aliquots of beads can be removed every 1-2 days and cell number and viability enumerated by the method of Goodwin et al, Proc. Soc. Exp. Biol. Med. 202:181 (1993)). Aggregate formation can be assessed by visual inspection under light microscopy and scanning electron microscopy. To determine the status of epithelial cell differentiation, indirect immunofluorescent studies can be performed with mAbs STE1, STE2, A3D8 and 11.24 (Patel et al, Int. Immunol. 7:277 (1995)) on frozen sections of microcarriers or by indirect

immunofluorescence and flow cytometry on cells liberated from Cytodex-3 beads by collagenase treatment.

Table 1

Components of thymic epithelia (TE) feeding medium

Component	Final Concentration	Supplier
DMEM	67%	Gibco
F12	22%	Gibco
Fetal Clone II	5%	HyClone
Hydrocortisone	0.4 μg/ml	Calbiochem
Cholera toxin	10^{-10} M	Schwarz/Mann
Insulin	0.135 I.U./ml	Sigma
Adenine	0.18 μM	Sigma
Sodium pyruvate	110 μg/ml	Gibco
EGF*	11.2 ng/ml	Amgen
Fungizone	247 ng/ml	Gibco
Gentamicin	50 μg/ml	Whitaker

*Recombinant human EGF

One of the problems in growing TE cells has been the overgrowth of fibroblasts which divide more rapidly than TE cells in two-dimensional tissue culture

systems. Growth of TF and TE cells may be different in a low gravity setting than on planar surfaces as has been demonstrated in Example 1. The problem of fibroblast overgrowth can be circumvented by purging the system of TF by complement mediated lysis after treatment with the fibroblast-specific mAB 1B10 (Singer, J. Invest. Dermatol. 92:166 (1989)). Thymic fibroblasts are, however, stimulatory for TE cell growth. Thus, to avoid overgrowth of fibroblasts but retain their effects on TE cell proliferation and tissue architecture, TF and TE cells can be co-cultured at different ratios(1:1, 1:5 and 1:25) on Cytodex-3 microcarrier beads in the STLV to determine the optimal ratio of TF to TE cells. Cell number, viability and differentiation status can be assessed as above. TF cells can be differentiated from TE cells by reactivity with anti-keratin mAb AE-3 on cytopreps or sections or by flow cytometry using antibodies to CD104 and CD105. TE cells express cytoplasmic keratins and CD104 while TF do not. TF do express surface CD105. To assess tissue architecture, immunofluorescence can be performed on frozen sections of beads using mAbs AE3 (to label TE cells), TE7 (to label TF) and STE1 and STE2 (to assess differentiation status of TE cells)). Tissue structure can be analyzed by transmission electron microscopy which can easily differentiate TF from TE cells, as TE cells (but not TF) contain tonofilaments and desmosomes.

EGF, insulin and IL-6 are growth factors for human TE cells and combinations of these factors may improve the growth of TE cells. Le et al, J. Exp. Med. 174:1147 (1991) have proposed that TGF-alpha is a growth factor for TE cells and that TGF-beta may be inhibitory for TE cell growth. IL-8 may be an autocrine growth factor for TE cells or may influence the expression of surface molecules. To increase the rate of proliferation and longevity of TE cell cultures, EFG, FGF, TGF-alpha, TGF-beta, IL-6 and IL-8 can be used alone or in combination to supplement TE cell and/or fibroblast growth. Not only may these factors affect growth and differentiation, they may (like IFN-gamma) affect the expression of adhesion and MHC molecules involved in T cell development. The effect of these factors can be determined by indirect immunofluorescence and flow cytometry on the expression of surface molecules expressed on TE cells.

To establish that precursor or mature T cells migrate to the thymic stromal tissues grown in microgravity, thymic stromal grafts can be implanted under the renal capsule of SCID mice using techniques previously reported (Barry et al, J. Exp. Med. 173:167 (1991)). SCID mice can be treated with anti-asialo GM-1 to abrogate endogenous NK cell activity. The ability of autologous and allogeneic thymocytes to migrate to the grafts can be tested by IP injection of 50 x 10^6 thymocytes. Migration to grafts can be assessed by IF

using anti-human CD2, CD3, CD4, CD6, CD7 and CD8 mAbs (all specific to thymocytes at different stages of T cell development). It can be determined whether thymic stromal cell cultures grown in microgravity function to induce thymocyte differentiation *in vitro*. Immature populations of autologous thymocytes, both triple negative (CD3-4-8-, CD7+) and double positive ($CD3^{lo}4+8+$), can be purified based upon expression of cell surface antigens by a combination of panning and fluorescence activated cell sorting (FACS) on the Becton-Dickinson FACStar^{Plus}. The immature thymocytes can be injected into autologous stromal tissues (grown *in vitro* in microgravity) using a Narishigi micromanipulator, and as a control into thymocyte-depleted chunks of autologous thymus. After *in vitro* culture, aliquots of tissues harvested at intervals up to 4 weeks can be analyzed for T cell differentiation by IF on frozen sections using mAbs to CD1, CD2, CD3, CD4, CD6, CD7 and CD8.

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All documents cited above are hereby incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of generating human dendritic cells comprising:

(i) explanting thymic cells from a human donor,

(ii) culturing the explanted cells from step (i),

(iii) exposing the cells resulting from step (ii) to human hematopoietic progenitor cells under conditions such that said progenitor cells develop into dendritic cells.

2. The method according to claim 1 wherein the cells resulting from step (ii) are present as a thymic stromal monolayer.

3. The method according to claim 1 wherein the cells resulting from step (ii) are present as a thymic stromal nodule.

4. The method according to claim 1 wherein, in step (iii), said cells resulting from step (ii) are contacted with said progenitor cells.

5. The method according to claim 1 wherein said culturing of said (ii) is effected under conditions such that T-cells present in said explant are depleted.

6. The method according to claim 1 wherein said exposing is effected *in vitro*.

7. The method according to claim 1 wherein said exposing is effected *in vivo*.

8. The method according to claim 1 wherein said progenitor cells are obtained from umbilical cord blood, bone marrow or peripheral blood.

9. The method according to claim 1 wherein said progenitor cells are lin^- cells.

10. The method according to claim 9 wherein said progenitor cells are $CD34^+lin^-$ cells.

11. A method of generating human dendritic cells comprising exposing immortalized human thymic epithelial cells to human hematopoietic progenitor cells under conditions such that said progenitor cells develop into said dendritic cells.

12. The method according to claim 11 wherein said thymic epithelial cells comprise a vector encoding papilloma virus E6E7 genes.

13. The method according to claim 11 wherein said thymic epithelial cells are contacted with said progenitor cells.

14. A human thymic epithelial cell comprising a vector encoding papilloma virus genes.

15. The human thymic cell according to claim 14 wherein said papilloma virus genes are E6E7 genes.

16. The human thymic cell according to claim 14 wherein said cell is a TE750 cell.

17. A dendritic cell generated by the process according to claim 1 or 11.

18. A method of treating or preventing an autoimmune disease comprising:

(i) introducing into dendritic cells according to claim 17 an antigen to which an immune response is directed in said disease, or nucleic acid encoding said antigen, under conditions such that said antigen is presented on the surface of said dendritic cells, and

(ii) introducing said dendritic cells resulting from step (i) into a patient in need of said treatment or prevention under conditions such that said treatment or prevention is effected.

19. The method according to claim 18 wherein said autoimmune disease is diabetes or multiple sclerosis.

20. A method of treating or preventing cancer comprising:

i) introducing into dendritic cells according to claim 17 a tumor antigen, or nucleic acid encoding said antigen, under conditions such that said antigen is presented on the surface of said dendritic cells, and

(ii) introducing said dendritic cells resulting from step (i) into a patient in need of said treatment or prevention under conditions such that said treatment or prevention is effected.

21. A method of preventing rejection of a graft by a host comprising:

(i) introducing into dendritic cells according to claim 17 an immunoreactive antigen, or nucleic acid encoding said antigen, of said graft under conditions such that antigen is presented on the surface of said dendritic cells, and

(ii) introducing said dendritic cells resulting from step (i) into said host under conditions such that said prevention is effected.

22. A method of treating or preventing a disease resulting from infection of a patient with a pathogen comprising:

(i) introducing into dendritic cells according to claim 17 an antigen, or nucleic acid encoding said antigen, of said pathogen under conditions such that said antigen is presented on the surface of said dendritic cells and

(ii) introducing said dendritic cells resulting from step (i) into said patient under conditions such that said treatment or prevention is effected.

23. The method according to claim 22 wherein said pathogen is a virus or a microorganism.

24. The method according to claim 23 wherein said pathogen is CMV, EBV or HIV.

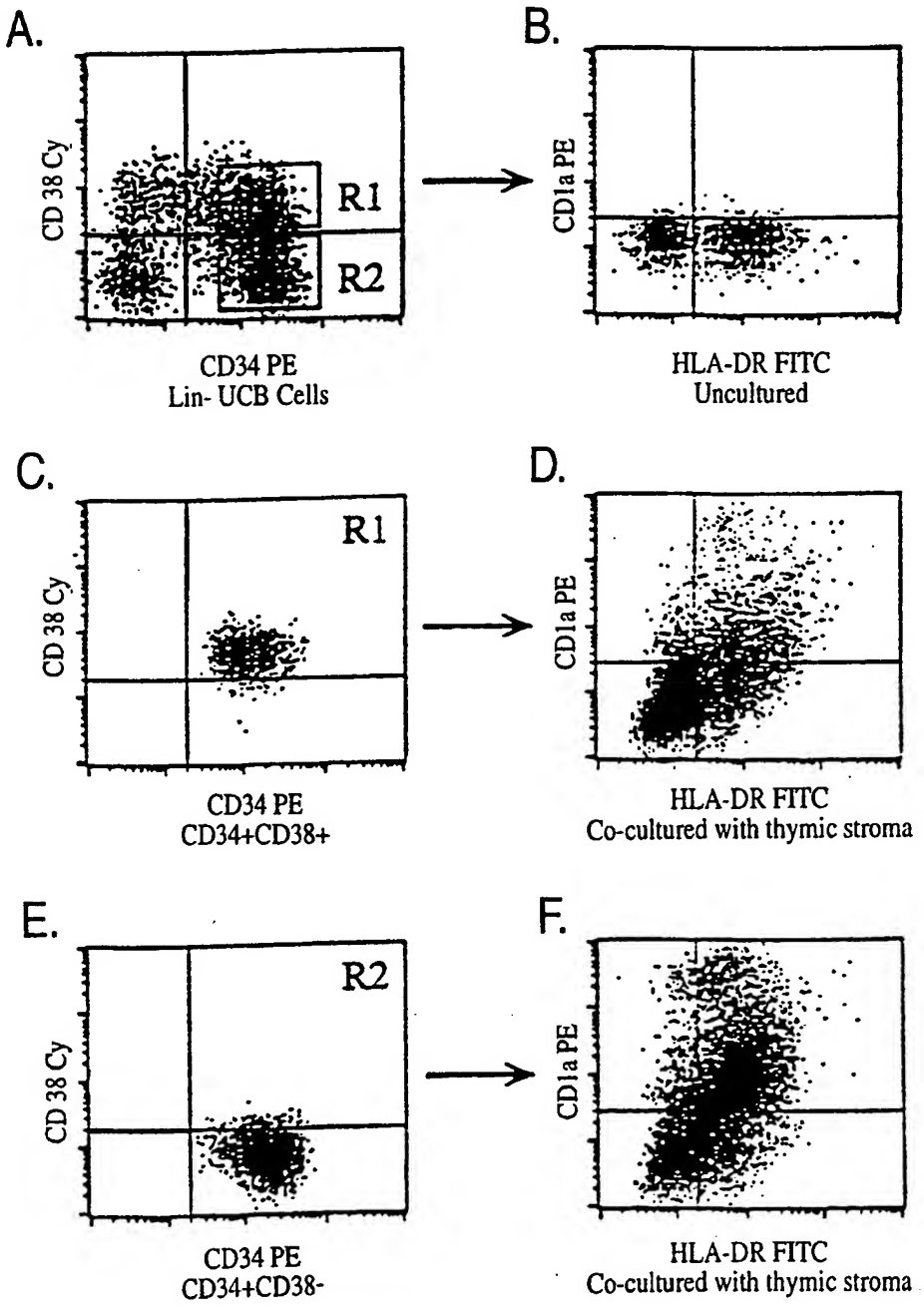
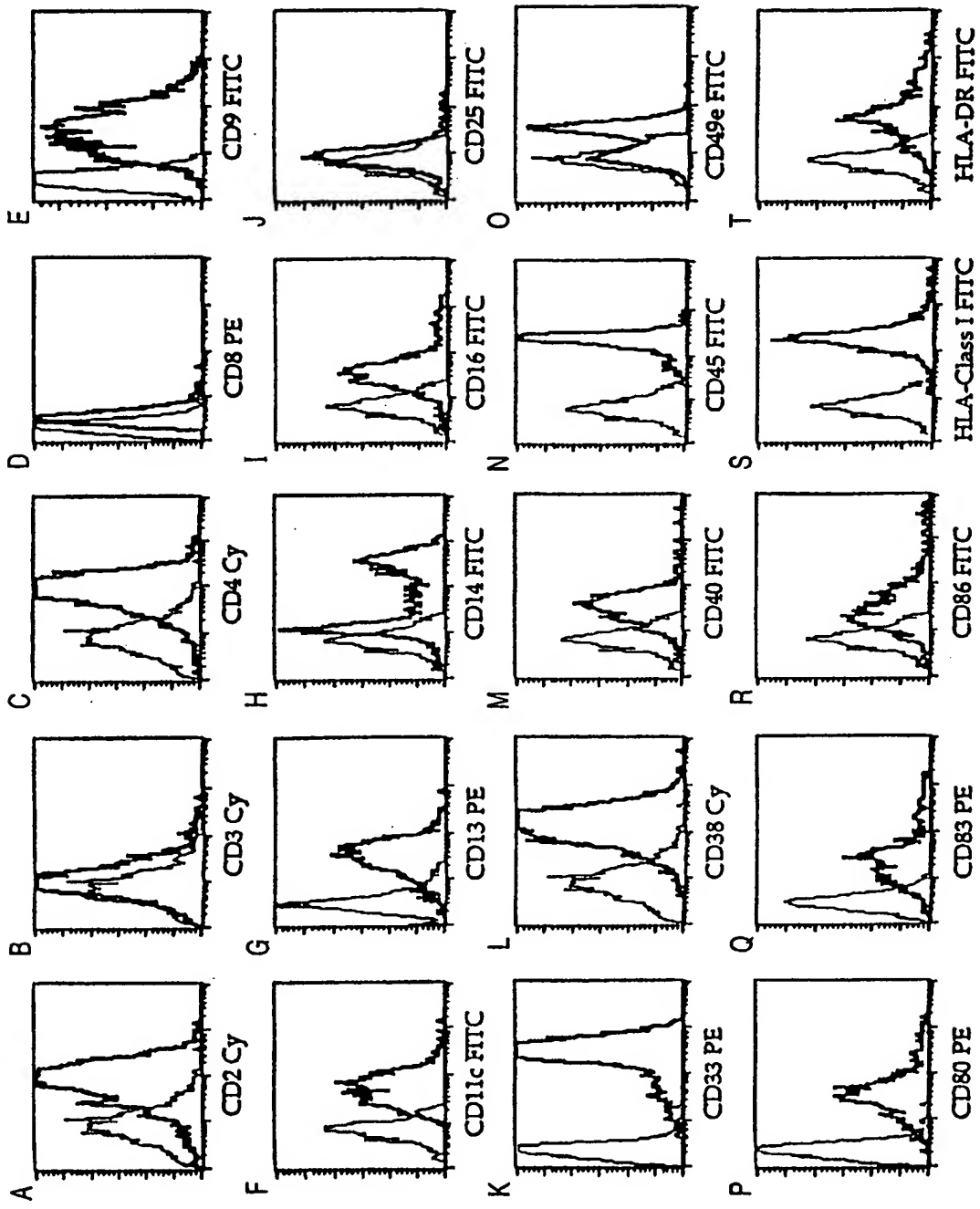

A.
B.
CD 38 Cy
R1
R2
CD34 PE
Lin- UCB Cells
CD1a PE
HLA-DR FITC
Uncultured
C.
D.
R1
CD 38 Cy
CD34 PE
CD34+CD38+
CD1a PE
HLA-DR FITC
Co-cultured with thymic stroma
E.
F.
R2
CD 38 Cy
CD34 PE
CD34+CD38-
CD1a PE
HLA-DR FITC
Co-cultured with thymic stroma

Fig.1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CD2 Cy
B
CD3 Cy
C
CD4 Cy
D
CD8 PE
E
CD9 FITC
F
CD11c FITC
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H
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I
CD16 FITC
J
CD25 FITC
K
CD33 PE
L
CD38 Cy
M
CD40 FITC
N
CD45 FITC
O
CD49e FITC
P
CD80 PE
Q
CD83 PE
R
CD86 FITC
S
HLA-Class I FITC
T
HLA-DR FITC

Fig.2

Fig.3B

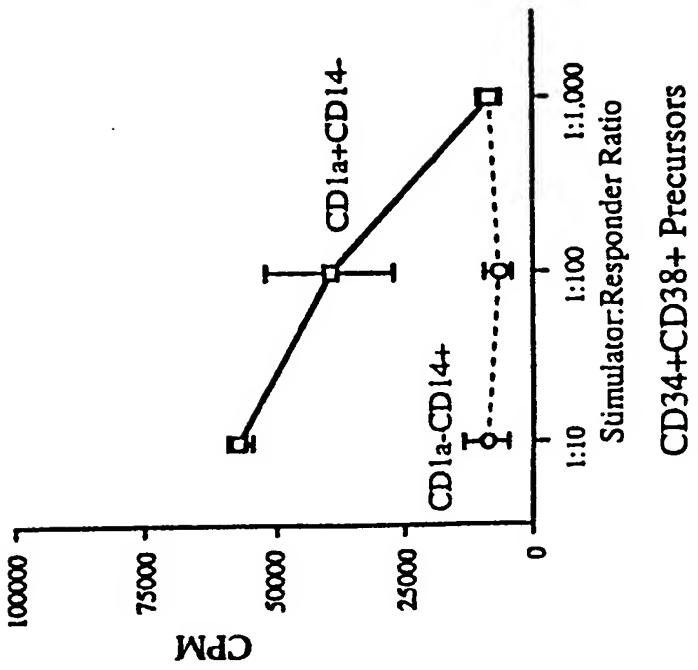
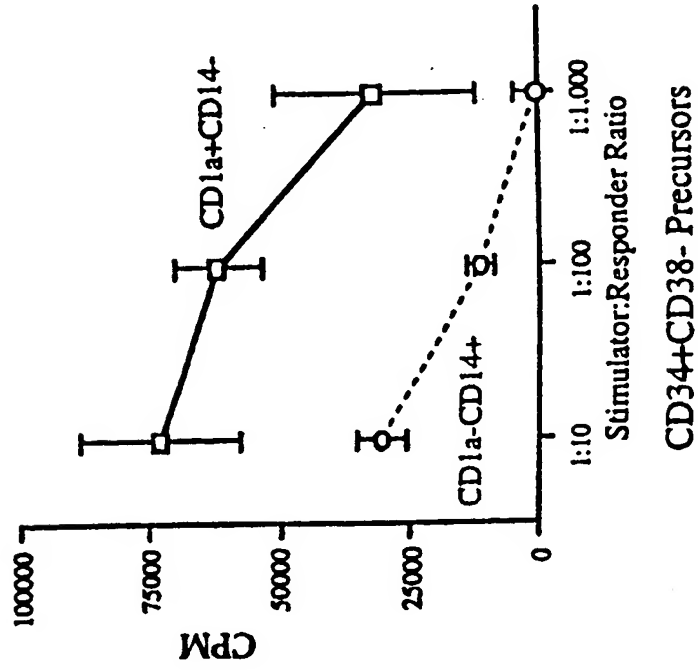

CPM
100000
75000
50000
25000
0
CD1a+CD14-
CD1a-CD14+
1:10
1:100
1:1,000
Stimulator:Responder Ratio
CD34+CD38+ Precursors

Fig.3A


CPM
100000
75000
50000
25000
0
CD1a+CD14-
CD1a-CD14+
1:10
1:100
1:1,000
Stimulator:Responder Ratio
CD34+CD38- Precursors

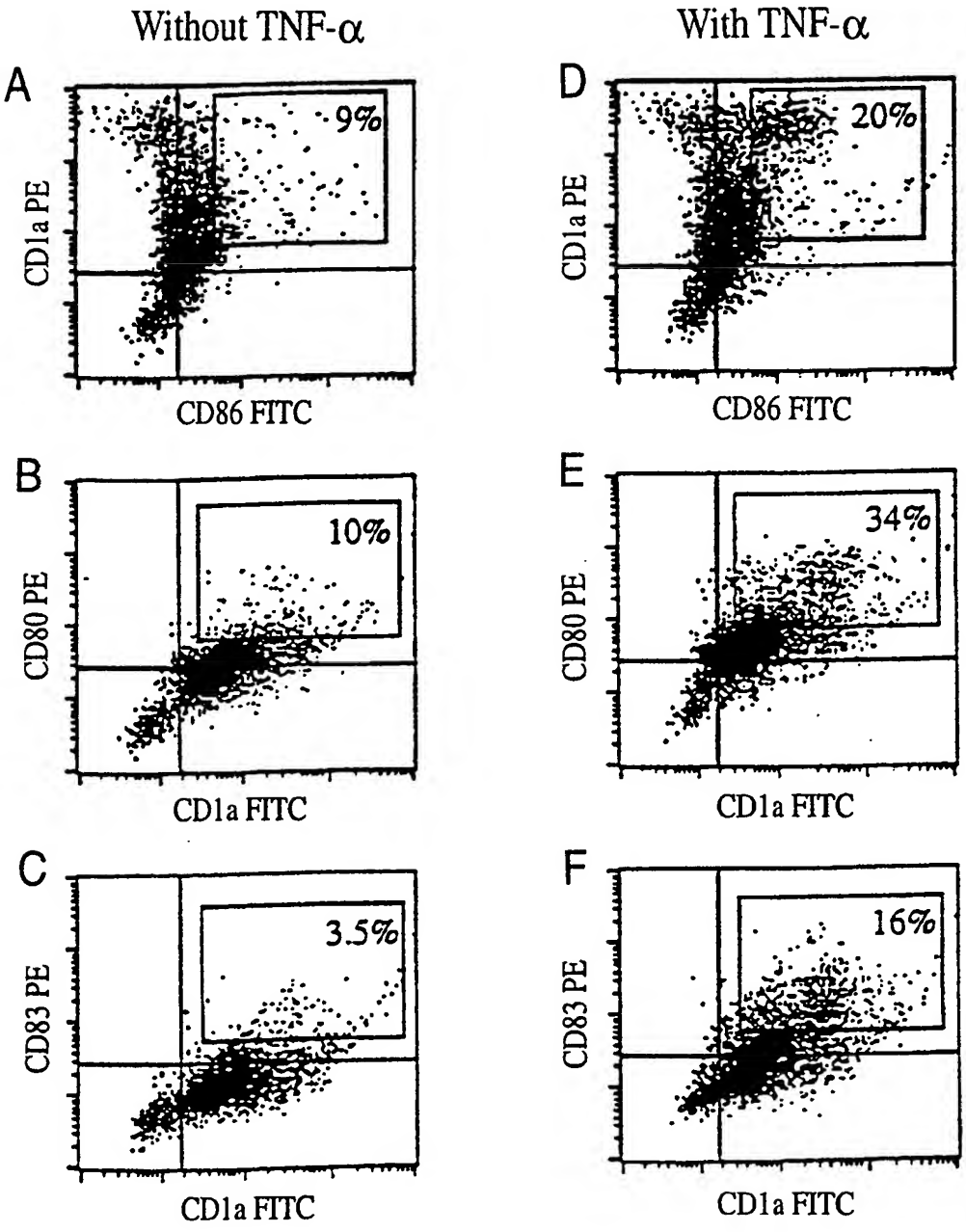

Without TNF-α
With TNF-α
A
9%
CD1a PE
CD86 FITC
D
20%
CD1a PE
CD86 FITC
B
10%
CD80 PE
CD1a FITC
E
34%
CD80 PE
CD1a FITC
C
3.5%
CD83 PE
CD1a FITC
F
16%
CD83 PE
CD1a FITC

Fig.4

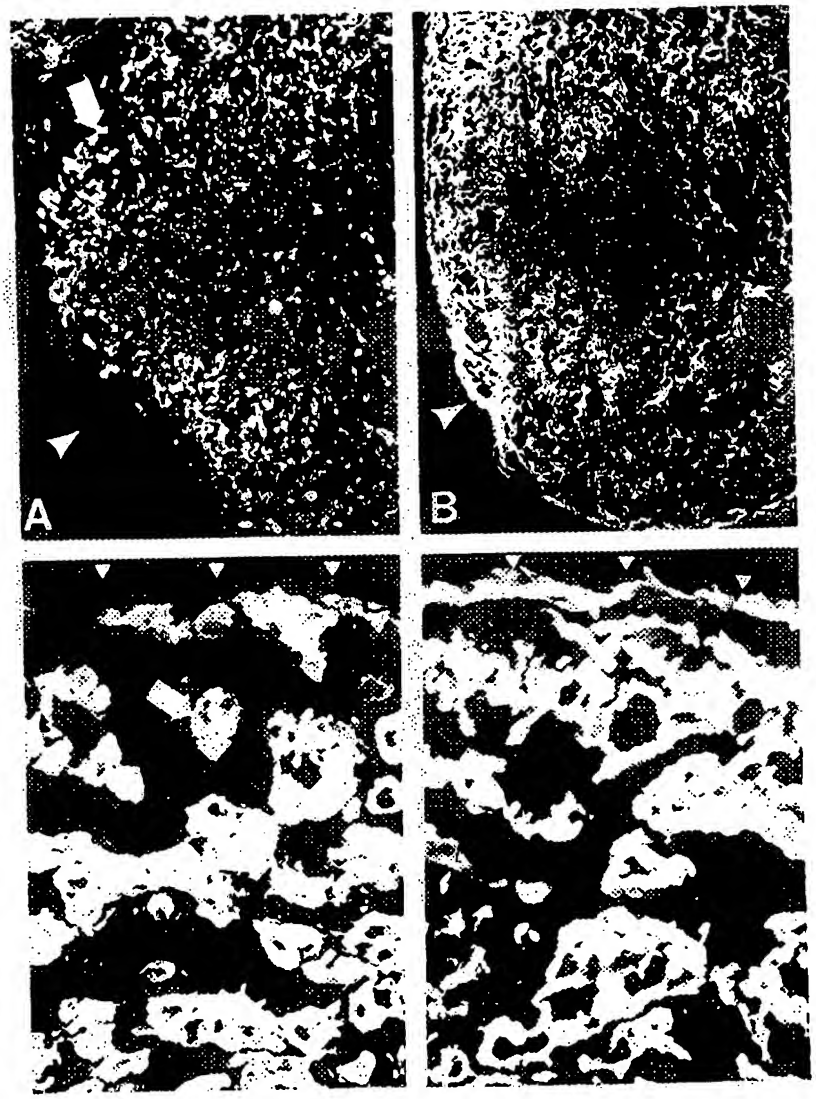


Fig.5

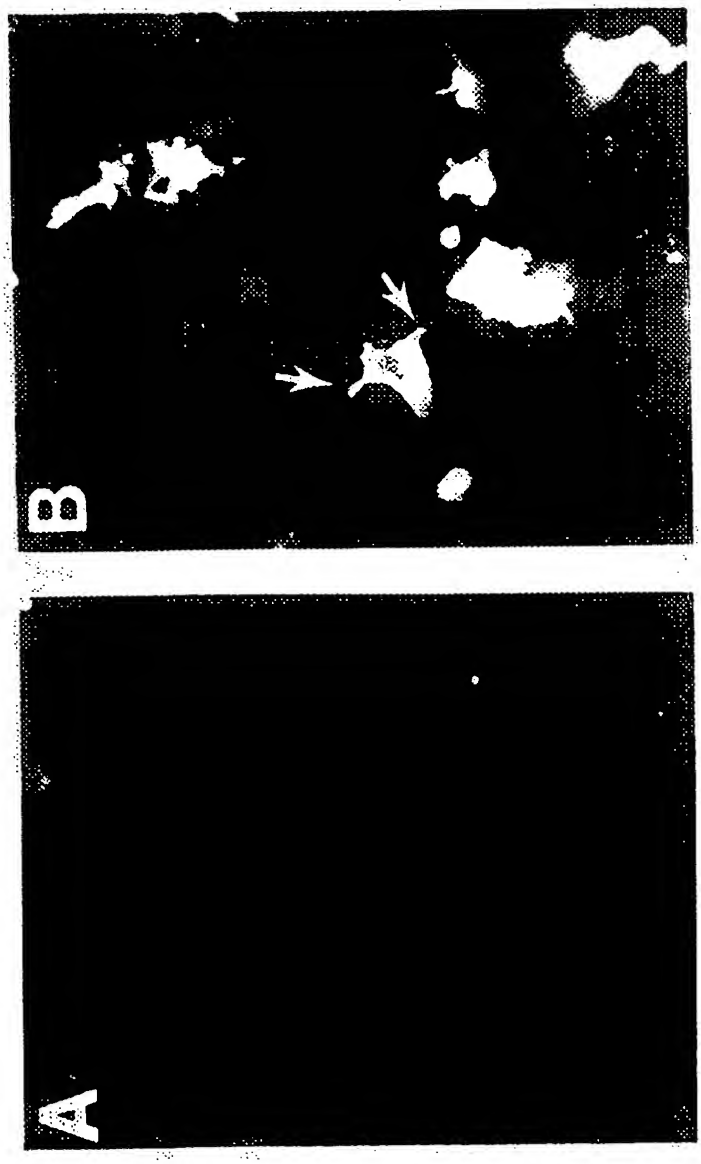


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18317

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00; A01N 63/00, 65/00
US CL :435/368, 371, 377, 378; 424/93.21, 93.7

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/368, 371, 377, 378; 424/93.21, 93.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Chemical Abstracts, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARDAVIN, C. et al. Thymic Dendritic Cells and T Cells Develop Simultaneously in the Thymus from a Common Precursor Population. Nature. 22 April 1993, Vol. 362, pages 761-763, especially page 762.	1-24
Y, P	SAUNDERS, D. et al. Dendritic Cell Development in Culture from Thymic Precursor Cells in the Absence of Granulocyte/ Macrophage Colony-Stimulating Factor. Journal of Experimental Cell Research. December 1996, Vol. 184, pages 2185-2196, especially pages 2187-2192.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
23 DECEMBER 1997

Date of mailing of the international search report
26 JAN 1998

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18317

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TSAO, S-W et al. Characterization of Human Ovarian Surface Epithelial Cells Immortalized by Human Papilloma Viral Oncogenes (HPV-E6E7 ORFs). Experimental Cell Research. 1995, Vol. 218, pages 499-507, especially pages 502, 505 and 506.	11-24
Y	MAYORDOMO, J. I. et al. Bone Marrow-Derived Dendritic Cells Pulsed with Synthetic Tumor Peptides Elicit Protective and Therapeutic Antitumor Immunity. Nature Medicine. December 1995, Vol. 1, No. 12, pages 1297-1302, especially pages 1298-1299.	18-24